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Two sites of interaction of ferredoxin with thylakoids

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The interaction of ferredoxin with thylakoids is shown to occur at two distinct sites at the reducing end of photosystem I, and at the site where ferredoxin-NADP reductase (FNR) is located on the membrane The evidence is based on the lack of inhibition of ferredoxin photoreduction by the extraction of FNR or its inactivation by an antibody, and on the difference between K_m values for ferredoxin in reactions requiring FNR as compared to those only requiring ferredoxin

Photosynthesis *NADP reduction* *Ferredoxin* *Ferredoxin-NADP reductase* *Photosystem I*

1. INTRODUCTION

It has been well-known for a long time that ferredoxin (Fd) is a soluble protein, the addition of which to isolated, stroma-free thylakoids is an absolute requirement for NADP photoreduction. The next enzyme in the chain, the FAD-containing flavoprotein, ferredoxin-NADP reductase (FNR, EC 1.18.1.2) is membrane-bound and located on the outer surface of the membranes [1] in the stroma-exposed regions of thylakoids [2]. Ferredoxin and FNR are known to form a 1 1 complex when mixed in solution [3] and it has been recently shown that the complex is also formed with the same dissociation constant when FNR is in its native state, bound to the membranes [4]. The formation of the complex is thought to be a requirement for NADP reduction in chloroplasts, on the basis of experiments where Fd was chemically modified so as to be unable to form the complex with FNR [5].

Two possible modes of action of Fd in electron transport can be envisaged. According to the first, Fd would be reduced by photosystem I (PS I) at its reducing site on the surface of thylakoids and then diffuse in the reduced form to the site where FNR is bound to the membrane. Here Fd would be reoxidized after forming the complex with FNR. This 'shuttle' model is in contrast with a model, recently proposed, according to which Fd binds to FNR, so connecting this enzyme to PS I, to mediate electron transport from PS I to FNR-NADP [6]. Two important implications of this model are that Fd would be unable to accept efficiently electrons from PS I unless it is bound to FNR, and PS I and FNR should be located very close to each other on the thylakoid membrane, so as to be connected by such a small protein as Fd of 10 kDa. According to this model, the linear electron transport from water to NADP would therefore not require the shuttle of Fd in solution from PS I to FNR, at the reducing side of the photosystem [6].

We have investigated this problem and report here results indicating that the Fd interaction with PS I is independent of its binding to FNR. This conclusion is reached on the basis of the observation that an antibody specific to FNR does not affect the Fd-dependent photoreduction of cytochrome (cyt.) *c*, while inhibiting completely the reduction of NADP. Furthermore, the extraction of FNR from thylakoids leads to inhibition of NADP reduction but does not affect cyt.*c* reduction. Evidence is also presented that the kinetic parameters indicating the interaction of Fd with

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the membranes in the cyt *c* photoreduction system and in NADP photoreduction are not consistent with the concept of a unique site for ferredoxin.

2. METHODS

Chloroplasts were prepared as described in [4], from freshly harvested spinach leaves. The photosynthetic reactions of stroma-free thylakoid suspensions were measured in a buffer containing 30 mM Tricine, pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$. This concentration of Mg^{2+} is saturating for its effects on Fd-dependent reactions [7,8]. In the case of NADP reduction 1 mM NADP was added, and Fd in the concentrations indicated. Cyt *c* reduction was measured under the same conditions, except for the omission of NADP and the addition of 100 μM horse heart cyt *c* (oxidized) and 4 mM NaN_3 The photochemical reactions were measured in a Sigma ZWS dual-wavelength spectrophotometer, equipped with a side illumination apparatus. NADPH formation was estimated as the absorbance increase at 340 minus 390 nm, and cyt.*c* reduction at 550 minus 540 nm. The actinic light, of saturating intensity, was filtered through a broad band red filter and a heat filter. Reduction of cyt *c* in the dark was measured in the presence of 1 mM NADPH, 5 mM glucose 6-phosphate and glucose-6-phosphate dehydrogenase in large excess. All activities are expressed as specific activities, in μequivalent electron mg^{-1} $Chl \cdot h^{-1}$.

The antibody against FNR was prepared according to [2], and the IgG fraction was obtained by ammonium sulfate fractionation of rabbit serum. The same fraction from non-immune serum served as a control, and had no influence on any of the reactions.

3. RESULTS

The effects of the antibody against FNR on NADP photoreduction and on cyt.*c* photoreduction are shown in table 1. While NADP reduction was progressively inhibited by increasing amounts of antibody, the reduction of cyt.*c* was unaffected. Similar results have been reported by Shahak et al. [9]. The experiment also shows that the antibody, raised against pure FNR, had no contaminating activities against other components of the electron transport system of thylakoids, as the 2 reactions measured share a common pathway of electron flow from water to Fd. Only at the last step, the reoxidation of reduced Fd, do the 2 reactions differ: NADP reduction involves the interaction of reduced Fd with membrane-bound FNR, while the reduction of cyt.*c* only requires the direct interaction of Fd with cyt.*c*, both molecules being in solution.

Table 1

Lack of effect of antibody against FNR on electron transport from H_2O to Fd

Antibody added (μl)	NADP photoreduction (μequiv mg^{-1} Chl h^{-1})	Cyt *c* photoreduction (μequiv mg^{-1} Chl h^{-1})
0	732	532
1	348	508
4	0 0	590

Conditions (see section 2) 20 μM ferredoxin, uncoupler, 2 μM gramicidin D; thylakoids contained 10 μg chlorophyll

Table 2 shows that the extraction of FNR from the membranes does not affect cyt *c* photoreduc-

Table 2

Effect of FNR extraction from the membranes on NADP and cyt *c* photoreduction

Reaction	Treatment of thylakoids	
	Extraction with buffer[a]	Trypsin
NADP reduction	35 4 ± 11	18 4 ± 1 1
Cyt *c* reduction	105 ± 17	94 ± 10
Diaphorase activity	15 6 ± 6 7[b]	22 ± 2[b]

[a] Thylakoids (500 μg $Chl \cdot ml^{-1}$) were extracted 3 times at 20°C for 10 min with Tricine NaCl-$MgCl_2$ buffer (see section 2) containing 10 mg/ml of bovine serum albumin, and sedimented by centrifugation at 0°C

[b] The activity lost by thylakoids was recovered in the medium after sedimentation of the membranes (see also [10])

The values (expressed as % of control activity) were averaged from 8 experiments, ± SE

tion, while inhibiting NADP reduction. The extraction was performed by 2 different procedures: mild trypsin treatment as described in [10] or successive extractions of the membranes with the Tricine-NaCl-$MgCl_2$ buffer used as the reaction medium.

Further evidence of the independent interaction of Fd with thylakoids at 2 different sites was obtained through the measure of kinetic parameters of the 2 Fd-dependent reactions. NADP and cyt.*c* photoreductions were measured, as a function of Fd concentration, at saturating concentration of NADP or, respectively, cyt.*c*, under light saturation conditions. Table 3 shows the difference in K_m for Fd of the 2 reactions. The values 0.67 and 0.087 μM were found for NADP and cyt.*c* photoreduction, respectively, the two V_{max} values being similar. When the thylakoids were uncoupled, the increase in V_{max} was considerably larger in the case of NADP reduction than cyt.*c* reduction (indicating that the latter reaction became rate limited at the reoxidation of reduced Fd by cyt.*c*, even if the concentration of cyt.*c* was apparently saturating), and the K_m for Fd increased to 3 μM in the case of NADP reduction, as reported in [11], and to 0.189 μM in the case of cyt.*c* reduction (table 3).

The relevant difference of K_m (Fd) of the 2 reactions was also observed when the maximal reaction rates were kept approximately equal through the partial inhibition of NADP reduction by the antibody to FNR (not shown).

In the above experiments, the interaction of Fd with the FNR site on the membrane involved reduced Fd, while at the PS I site oxidized Fd was involved. We have therefore investigated a reaction sequence where oxidized Fd functions as an electron acceptor at the FNR site, namely the sequence NADPH⟶FNR⟶Fd⟶cyt.*c*. This sequence is known to be inhibited by NADPH at low Fd concentration [12]. Therefore, the reciprocal maximal reaction rates obtained from Lineweaver-Burk plots of velocity against NADPH concentration, extrapolated to infinite NADPH concentration from the linear part of the plots obtained at different Fd concentrations (fig 1), were plotted against 1/[Fd] (fig.1, inset). The observed K_m for Fd was 2.2 μM (with a V_{max} of 110 μequiv./mg Chl

Table 3

Kinetic parameters of NADP and cyt *c* reduction by thylakoids

	Electron transport system			
	H_2O to NADP		H_2O to cyt *c*	
	K_m Fd (μM)	V_{max}	K_m Fd (μM)	V_{max}
Coupled	0.63	244	0 071	238
	0 71	286	0 103	243
mean:	0 67	265	0 087	240
Uncoupled	3 12[a]	832[a]	0 200[a]	400[a]
	2 91[b]	832[b]	0.179[b]	357[b]
mean	3.01	832	0 189	378

Conditions: see section 2 Uncoupler: [a] 2 μM gramicidin, [b] 5 mM NH_4Cl Both experiments were run in duplicate 10 μM chlorophyll, 1 mM NADP. K_m (Fd) and V_{max} were calculated from Lineweaver-Burk plots, the regression coefficient of the straight lines being 0 99 or better

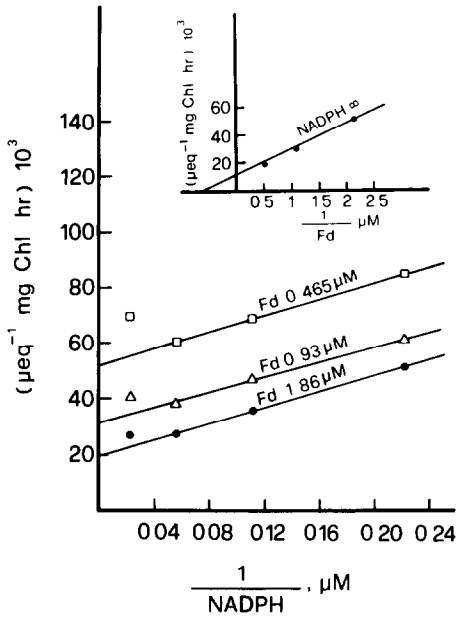

Fig 1. Lineweaver-Burk plot of NADPH-cyt.*c* reductase of thylakoids versus NADPH concentration, at different Fd concentrations Inset the ordinate intercepts at infinite NADPH concentration are plotted against 1/[Fd] 9 2 μM chlorophyll

per h), i.e. of the order of that observed in the case of NADP photoreduction, and considerably larger than that of the cyt.*c* photoreduction reaction (table 1).

4. DISCUSSION

Our experiments, in agreement with the findings by Shahak et al. [9], show that the antibody specific to FNR does not affect electron transport from water to Fd, as indicated by the lack of inhibition of Fd-dependent cyt.*c* reduction, though inhibiting NADP reduction. Our experiments show that the extraction of FNR from the membranes (table 2, see also [10]) or its inactivation by a specific antibody (table 1), inactivate NADP photoreduction by thylakoids, but do not affect the Fd-dependent cyt.*c* photoreduction. These findings are in agreement with [9,10].

Our interpretation is that the reduction of Fd by PS I does not require the binding of Fd to the membrane-bound FNR as postulated in [6], but involves an independent site of interaction at the reducing end of PS I. The existence of 2 different sites for Fd on the membranes is also demonstrated by the quite different values of K_m for Fd of the reactions involving only Fd (such as the photoreduction of cyt.*c*) as compared to the reactions involving both Fd and FNR, such as NADP photoreduction and the Fd-dependent NADPH-cyt *c* reductase activity of thylakoids.

Another important indication derived from the lack of inhibition by the antibody to FNR of Fd reduction is that the reducing site of PS I should be far enough apart from FNR as to prevent steric hindrance inhibition of the Fd-PS I interaction by a large molecule such as a 7 S IgG molecule, of approx. 120–150 Å diameter.

Our observations are therefore consistent with the role of Fd as a shuttle for electrons from PS I to FNR in the process of NADP photoreduction.

REFERENCES

[1] Berzborn, R (1969) Z Naturforsch 24B, 436–446
[2] Jennings, R C., Garlaschi, F M , Gerola, P D and Forti, G (1979) Biochim Biophys Acta 546, 207–219.
[3] Foust, G.P , Mayhew, S G. and Massey, V (1969) J Biol Chem 244, 964–970
[4] Forti, G and Bracale, M (1984) FEBS Lett 166, 81–84
[5] Davis, D J and San Pietro, A (1977) Biochem Biophys. Res Commun 74, 33–40
[6] Carrillo, N and Vallejos, R H (1983) Trends Biochem Sci 8, 52–56
[7] Harnischfeger, G and Shavit, N (1974) FEBS Lett 45, 286–289
[8] Olsen, L F and Cox, R P (1980) Photobiochem Photobiophys 1, 147–153
[9] Shahak, Y , Crowther, D and Hind, G (1981) Biochim Biophys. Acta 636, 234–243
[10] Forti, G , Cappelletti, A , Nobili, R L , Garlaschi, F M., Gerola, P D and Jennings, R C (1983) Arch Biochem. Biophys 221, 507–513
[11] Carrillo, N , Lucero, H A and Vallejos, R H (1981) J Biol Chem 256, 1058–1059
[12] Forti, G and Sturani, E (1968) Eur J Biochem 3, 461–472